

REMARKS

The Advisory Action of March 14, 2005 indicated that the November 17, 2004 Amendment did not place the instant application in condition for allowance on two separate grounds.

First, the Declaration of Michael Hamblin is stated to “admit” that the 4 sentences at column 7, lines 18-30 of U.S. 5,552,452 (“the Khadem patent”) are directed to methods that do not require the application of exogenous substrates. Whether or not such methods are disclosed is not in question. Rather, the relevant question concerns only whether such methods are enabled. Proof of non-enablement depends on whether one of ordinary skill in the art could have combined the publication’s description of the invention with his own knowledge to make the claimed invention. If not, the reference is not enabled and therefore, removed from consideration under 35 U.S.C. § 102(b). See M.P.E.P. § 2121.01; *Chester v. Miller*, 15 U.S.P.Q.2d 1333, 1336 (Fed. Cir. 1990); *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1354 (Fed. Cir. 2003); *In re Hoeksema*, 399 F.2d 269, 158 U.S.P.Q. 596 (CCPA 1968); *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1354 (Fed. Cir. 2003); *In re Sun*, 31 U.S.P.Q.2D 1451, 1452 (Fed. Cir. 1993), *In re Donohue*, 766 F.2d 531 (Fed. Cir. 1985); *In re Sasse* 629 F.2d 675, 681 (C.C.P.A. 1980), and *In re Borst*, 345 F.2d 851, 855 (C.C.P.A. 1962). Thus, mere recitation of the methods at column 7, lines 18-30 is not enough to justify citation of the Khadem patent against the instant application under § 102(b); such methods must also be enable

The declaration of Dr. Michael Hamblin submitted on April 12, 2004 (“the Hamblin Declaration”) has provided substantial evidence to support the Applicant’s conclusion that the methods at column 7, lines 18-30 of the Khadem patent are not enabled. Yet, the Examiner has failed to provide any basis for disputing Dr. Hamblin’s conclusion that “the Khadem patent would not have provided one of ordinary skill in the art with the ability to combine its teachings with his own knowledge, in order to practice methods for adhering tissue in the absence of exogenous substrate.” See the April 12, 2004 Declaration of Dr. Michael Hamblin, paragraph 5, last sentence. It is respectfully requested that the Hamblin Declaration be reconsidered as rebuttal evidence under 35

U.S.C. § 102(b) on the issue on non-enablement, with application of the standard adopted by the Office in M.P.E.P. § 2121.01.

Submitted herewith is a second declaration by Dr. Hamblin, which reaffirms and further clarifies his initial position with respect to non-enablement of the methods at column 7, lines 18-30 of the Khadem patent. In view of both declarations by Dr. Hamblin, and the arguments of record in Applicant's previous response dated November 17, 2004, reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(b) is respectfully requested.

Second, the Advisory Action dated March 14, 2005 indicated that the declaration of John Khadem, M.D. ("the Khadem Declaration"), submitted during the prosecution of the Khadem patent, did not provide further proof of non-enablement of the methods at column 7, lines 18-30 of the Khadem patent. The data in the Khadem Declaration was apparently deemed non-persuasive because the methods at column 7, lines 18-30 of the Khadem patent depend on the use of an endogenous substrate protein. Quite to the contrary, the results in the Khadem Declaration demonstrate that Dr. Khadem could not practice these very same methods.

Dr. Khadem administered four different photosensitizer and protein formulations, having either 18% fibrinogen, 0.9% fibrinogen, 0.27% alpha crystalline or 0.27% beta crystalline, to human cadaver eyes. After attempting photochemical bonding with each formulation, Dr. Khadem concluded that the applied formulations must contain at least 1% protein substrate in order to effectively bond tissue. In the three samples that failed to effectively bond—those receiving formulations having less than 1% exogenous protein substrate—endogenous protein substrates were present in the donor tissue (see the second declaration of Michael Hamblin, submitted herewith). Yet, these endogenous substrates apparently could not facilitate effective bonding in the experiments conducted by Dr. Khadem. Therefore, having read the Khadem Declaration, one skilled in the art would conclude that Dr. Khadem could not practice the methods at column 7, lines 18-30 of the Khadem patent.

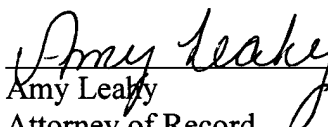
In view of all evidence of record, reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(b) is respectfully requested.

CONCLUSION

In view of the amendments and remarks herewith, the application is in condition for allowance. Favorable reconsideration of the application, reconsideration, and withdrawal of the objections to and rejections of the application, and prompt issuance of a Notice of Allowance are respectfully requested.

Respectfully submitted,

June 15, 2005



Amy Leaky
Attorney of Record
Reg. No. 47, 739
EDWARDS & ANGELL, LLP
Intellectual Property Practice Group
P.O. Box 55874
Boston, MA 02205
Telephone: (203) 353-6817

Differential Reactivity of Upper Triplet States Produces Wavelength-Dependent Two-Photon Photosensitization Using Rose Bengal

Christopher R. Lambert,[†] Irene E. Kochevar,[‡] and Robert W. Redmond^{*‡}

Chemistry Department, Connecticut College, 270 Mohegan Avenue, New London, Connecticut 06320, and Wellman Laboratories of Photomedicine, Department of Dermatology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114

Received: September 22, 1998

Two-photon laser flash photolysis was used to investigate the wavelength dependence of upper excited state chemistry of Rose Bengal (RB) in order to rationalize differences in RB-sensitized inhibition of acetylcholinesterase (ACE) in red blood cell ghosts, observed using different irradiation modes. The absorption of the RB triplet state, generated using 532-nm irradiation, was monitored as a function of wavelength used for subsequent excitation to upper triplet levels. Triplet state bleaching, due to formation of radical species, was observed using wavelengths greater than ~580 nm to excite into an upper state, T_n . However, no bleaching was observed at lower wavelengths, corresponding to higher photon energies, but an increase in emission intensity from the sample, consistent with $S_1 - S_0$ fluorescence, was observed following the second pulse. A mechanism for this wavelength-dependent photochemistry is provided, which considers the relative positions of upper singlet and triplet states. Reverse intersystem crossing, and hence repopulation of the triplet state, are only observed when the energy of the second photon is sufficient to access a triplet state (T_{n+1}) of similar energy to the S_2 state. At lower photon energies (higher wavelengths), a lower energy state (T_n) state is accessed, from which radical formation and bleaching of the triplet state ensues.

Introduction

There has been increasing interest in photobiology in the use of pulsed lasers to facilitate photochemical reactions that would not be observed under continuous wave (CW) irradiation, the high peak intensity of the pulsed irradiation being sufficient to initiate sequential two-photon absorption events and the population of upper excited states from which different chemical reactions might occur.^{1–8} Under continuous-wave visible irradiation, reactions are generally confined to those mediated by the lowest excited states of the photosensitizer, in particular the generation of singlet oxygen ($O_2(^1\Delta_g)$) by energy transfer from the lowest excited triplet state (T_1) to oxygen. Previous experiments from our laboratory have investigated the effects of upper state excitation in the xanthene-type photosensitizer, Rose Bengal (RB).^{5,9,10} A study of the wavelength dependence of RB-sensitized inhibition of acetylcholinesterase (ACE) in red blood-cell ghosts revealed that, while excitation of the $S_0 \rightarrow S_1$ transition by visible light gave rise to only singlet oxygen mediated type II photosensitization, the use of UVA light to excite an $S_0 \rightarrow S_n$ transition gave enhanced inhibition, attributed to the observed reactive radical formation from a S_n state.⁹

An alternate route to excitation into upper states is via sequential absorption of two lower energy (visible) photons, with the second photon being absorbed by a transient intermediate species. This can be achieved using a single high-intensity pulse, but upper state processes will only be observed when the S_1 or T_1 state absorbs competitively with the ground state precursor at the excitation wavelength.¹¹ Alternatively, and much

more selectively, a two-color, two-photon strategy can be used where advantage is taken of the spectral differences between ground state and intermediate to ensure selective excitation takes place in each case. The sequential absorption of two photons to populate higher excited states of organic molecules and facilitate reaction mechanisms that are not normally observed (reluctant) from the lowest excited states is well established.¹²

In a subsequent publication the sequential two-color, two-photon approach was used to excite into an upper triplet state using a 640-nm dye laser pulse to excite T_1 , following its formation by 532-nm excitation of the S_0 ground state and intersystem crossing. Comparison of one- and two-photon excitation showed an enhanced inhibition of ACE and enhanced phototoxicity to mouse macrophage monocyte P388D1 cells on upper triplet state formation by this route.⁵ Additionally, these effects did not require the presence of oxygen, ruling out the type II photooxidation mechanism. Concomitant time-resolved spectroscopic studies showed a bleaching of the triplet state accompanying the absorption of the second pulse and confirmed formation of radical species of RB that were responsible for the oxygen-independent phototoxicity.

With the sequential two-photon photosensitization route established, a subsequent investigation focused on sequential two-photon absorption and population of upper excited states of RB using a single, high-intensity 532-nm pulse.¹⁰ These studies gave somewhat puzzling results, as it was shown that even at the highest pulse energies used, where sequential two-photon absorption was indeed occurring, no oxygen-independent inhibition of ACE was observed, in contrast to the two-photon two-color studies, and the phototoxicity could be modeled purely on the basis of singlet oxygen formation.¹⁰

In the latter two studies, excitation of the T_1 state to upper triplet states occurs on absorption of a second photon, and thus,

* Corresponding author. Tel: 617-726-8857. Fax: 617-726-3192. E-mail: Redmond@helix.mgh.harvard.edu.

[†] Connecticut College.

[‡] Harvard Medical School.

similar results might have been expected for photosensitized inhibition of ACE. It was not clear why a type I photooxidation mechanism was not observed for the high-intensity, single pulse work, and the results suggested a difference in the photochemistry of RB when excited by these two methods. In the sequential, two-color, two-photon work a sequence of 532 + 640-nm pulses was used whereas the two-photon absorption in the high-intensity single pulse work was obviously 532 + 532 nm, the second pulse in each case being absorbed by the T_1 state. This work describes a series of experiments designed to investigate the photophysics of RB when excited by sequential absorption of two photons as a function of the wavelength of the second photon in an attempt to rationalize the observed differences in photobiological response.

Materials and Methods

Rose Bengal was obtained from Aldrich (Milwaukee, WI) with a stated purity of 97% and was used as received. Phosphate buffer (5 mM phosphate, pH = 7.4) was prepared using the appropriate mixtures of 5 mM KH_2PO_4 and 5 mM K_2HPO_4 , from Sigma (St. Louis, MO). The two-photon laser flash photolysis apparatus has been described previously.¹³ Briefly, the first excitation pulse was obtained from a frequency-doubled Quantel YG660A Nd:YAG laser (8 ns duration, 532 nm, Continuum, Santa Clara, CA). The second laser pulse was derived from a tunable optical parametric oscillator (MOPO 710, Spectra-Physics, Mountain View, CA) pumped by a Nd:YAG laser (Quanta Ray GCR 230, Spectra-Physics Mountain View, CA). Temporal synchronization of both sources was achieved using a Stanford Research Systems (Sunnyvale, CA) DG535 digital delay generator. Care was taken to ensure good spatial overlap of the two beams at the sample. The 10-mm path length quartz cuvette was sealed with a rubber septum, and nitrogen-saturated solutions were introduced into the cuvette via capillary tubing and a hypodermic needle. Continuously flowing samples were used in all experiments. The cuvette was masked such that the monitoring beam only passed through the front 1 mm \times 1 mm \times 3-mm volume of the cuvette.¹³ To ensure beam homogeneity a 3-mm cuvette was also used in some experiments with expanded laser beams. The detection system has been described in detail.¹⁴ In some experiments emission was detected from the sample using a gated, intensified, dual diode array, optical multichannel analyzer (OMA, Princeton Instruments DIDA 512G detector, ST120 controller and PG-10 pulse generator).¹³ To filter out scattered light from the lasers (532 and 640 nm) as much as possible, a combination of long-pass (10% transmittance at 550 nm) and short-pass (10% transmittance at 610 nm) filters was inserted between the sample and detector.

Results

Figure 1 shows the absorption spectra of the ground state (S_0), the triplet-singlet difference, and the corrected triplet spectrum (T_1) of RB in phosphate buffer at pH 7.4, as measured by absorption spectroscopy and laser flash photolysis.¹⁰ For two-color excitation using 532 + 640-nm pulses, the second photon is selectively absorbed by the T_1 state, as there is no ground state absorption for RB at this wavelength (Figure 1 inset). Two-photon laser flash photolysis of a 5 μ M RB solution ($A_{532} \sim 0.2$) was carried out as a function of the wavelength of the second photon. Figure 2 shows the transient absorption observed at 620 nm following laser flash photolysis using 532 + 640-nm excitation. The absorption of the 640-nm light pulse results in an instantaneous loss of T_1 absorbance (bleaching), similar

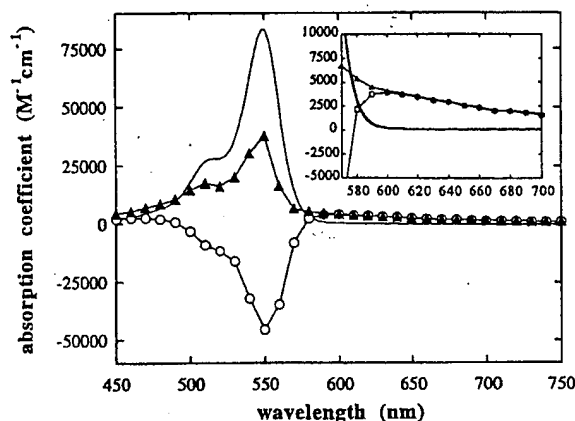


Figure 1. Ground state spectrum (—), triplet-ground state difference spectrum (○), and corrected triplet spectrum (▲) of RB in 5 mM phosphate buffer, pH 7.4. Inset: expanded region showing that the triplet state is selectively excited at 640 nm.

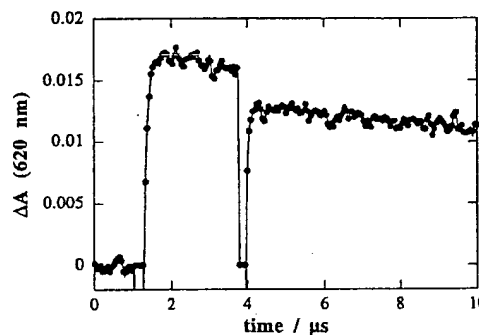


Figure 2. Time-dependent transient absorption monitored at 620 nm following sequential 532 + 640-nm excitation of 5 μ M RB in deaerated phosphate buffer, pH 7.4.

to that reported previously.⁵ In the previous work a flashlamp-pumped dye laser (pulse duration, $\tau_d = 400$ ns) was used to supply the second pulse, and although the wavelength was the same, the pulse duration is very different from that of the OPO ($\tau_d = 8$ ns) laser used in this study. The similarity in observed triplet bleaching in both studies indicates that the duration of the second pulse is not an important factor in determining the photochemical mechanism. The mechanism by which bleaching occurs was attributed to upper excited state photochemistry⁵ and indeed formation of long-lived radical species of Rose Bengal are detected by laser flash photolysis, absorbing at ~ 490 nm.

To carry out an experiment to mimic high-intensity single photon excitation, a two pulse 532 + 532-nm excitation protocol was used. As the absorption of the second pulse is now no longer spectrally selective (both S_0 and T_1 absorb at this wavelength), it was necessary to use sufficient energy in the first pulse to induce complete conversion of ground state to triplet state. This is possible as the quantum yield of triplet formation in RB is close to unity¹⁵ and as the S_1 lifetime of RB ($\tau = 95$ ps¹⁶) is much shorter than the pulse duration; cycling can occur to ensure all molecules are quickly converted to the long-lived T_1 state.¹⁷ Decay of the triplet state formed by the first pulse is minimal prior to the arrival of the second pulse; thus, virtually all molecules are in the T_1 state and absorption of the second 532-nm pulse is essentially selective by the T_1 state.

Figure 3 shows that the second 532-nm laser pulse causes an increase in the triplet absorption at 620 nm, rather than the decrease seen on 532 + 640-nm excitation. It is notable that

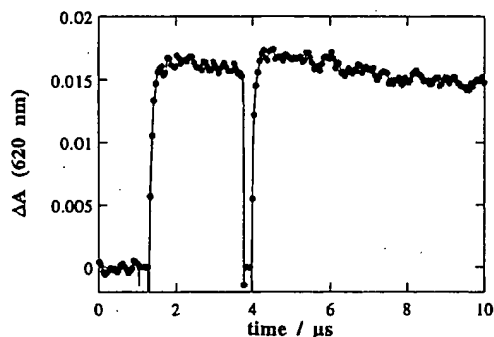


Figure 3. Time-dependent transient absorption monitored at 620 nm following sequential 532 + 532-nm excitation of 5 μ M RB in deaerated phosphate buffer, pH 7.4.

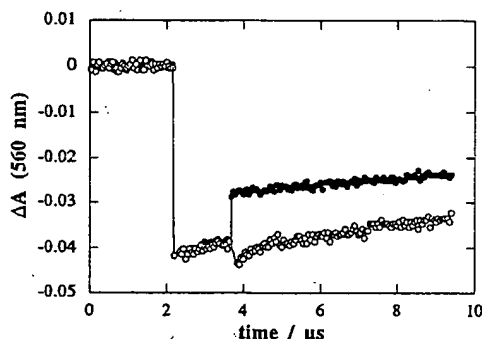


Figure 4. Time-dependent transient absorption monitored at 560 nm following (●) 532 + 620-nm and (○) 532 + 570-nm excitation of 5 μ M RB in deaerated phosphate buffer, pH 7.4.

the ΔA values immediately after both the first and second pulse is essentially identical, and the increase following the second pulse can therefore be rationalized by re-excitation of the small amount of RB molecules, which had relaxed from the triplet state back to the ground state prior to the second pulse. Thus, under conditions where complete conversion is achieved with the first laser pulse (using laser energies of up to 50 mJ/pulse), bleaching is not observed with a second 532-nm pulse. This experiment has also been carried out as a function of the delay time of the second pulse. It was not possible to observe bleaching of the T_1 state at 620 nm under any of the conditions used where 532 nm was used for the second pulse.

The results of a more detailed study of the wavelength dependence of the second pulse on photobleaching of the triplet state are shown in Figure 5. These experiments were also carried out under similar conditions except that the detection wavelength chosen for this experiment was 560 nm, in the bleaching region of the triplet difference spectrum, where signals are inverted but greater in magnitude. A 3-mm path length cuvette was used in these experiments to aid in ensuring complete overlap of laser beams on the probed volume of the sample. The wavelength of the second pulse from the OPO was varied, but the pulse energy remained constant at ~ 50 mJ/pulse. At wavelengths above 580 nm (e.g., 620 nm, Figure 4) the amount of bleaching of the T_1 state produced by the second pulse is constant and wavelength-independent. At wavelengths lower than 580 nm (e.g., 570 nm, Figure 4) the extent of bleaching decreases until an increase in transient absorption is seen with the arrival of the second pulse, consistent with re-excitation of the small fraction of molecules that had relaxed from the T_1 to S_0 state after the first pulse. Figure 5 shows the transient absorption change at 560 nm as a

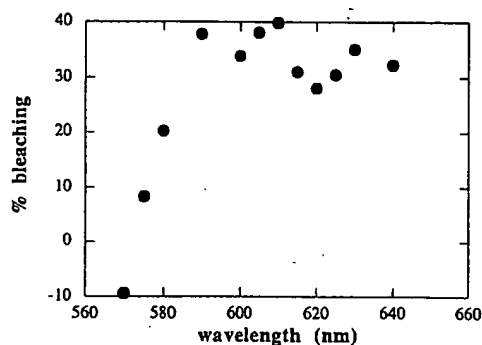


Figure 5. Dependence of fraction of bleached triplet absorption at 620 nm on the wavelength used for excitation of the T_1 state. Errors associated with these measurements are estimated at 15%.

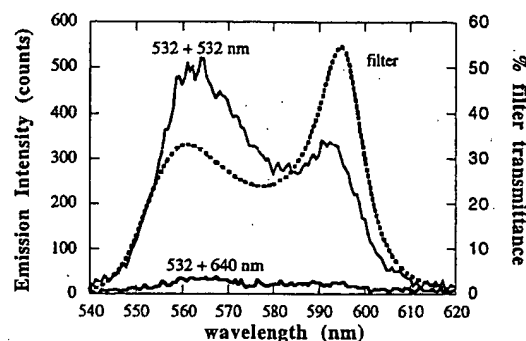


Figure 6. Fluorescence emission spectra recorded after the second pulse on 532 + 640-nm and 532 + 532-nm excitation of RB in deaerated phosphate buffer, pH 7.4. Also shown is the transmission profile (---) of the filter combination used to keep 532 and 640-nm laser light from reaching the detector.

function of the wavelength of the second pulse. Identical effects were seen using 620 nm as the detection wavelength.

Figure 6 shows the fluorescence emission at the time of the second pulse. Using a gated intensified diode array detector, it was possible to obtain the emission spectrum of this fluorescence that was found to be indistinguishable from the normal fluorescence spectrum of RB obtained on excitation of the ground state. Results from experiments using both 640- and 532-nm wavelengths for the second pulse are shown in Figure 6. The integrated area of fluorescence observed on 532-nm excitation of the T_1 state is a factor of 13 greater than that observed using 640 nm. The fluorescence emission spectrum has been somewhat altered from that normally seen in a fluorometer by the characteristics of the transmission spectrum of the filter combination used, shown in Figure 6.

Discussion

The observed photobleaching of the T_1 state of RB on 640-nm excitation confirms our earlier report that used a flashlamp-pumped dye laser to provide the 640-nm pulse. Thus, the differences between results on ACE photosensitization using the two-color and single pulse experiments were not due to a pulse duration effect.

Possible fates of a molecule excited into T_n are (i) rapid radiationless decay back to T_1 , (ii) upper excited state chemical reaction, as postulated by Smith et al.,⁵ and (iii) reverse intersystem crossing to the singlet manifold. The bleaching observed upon excitation with the second pulse provides evidence for pathway ii. This pathway does not appear to take

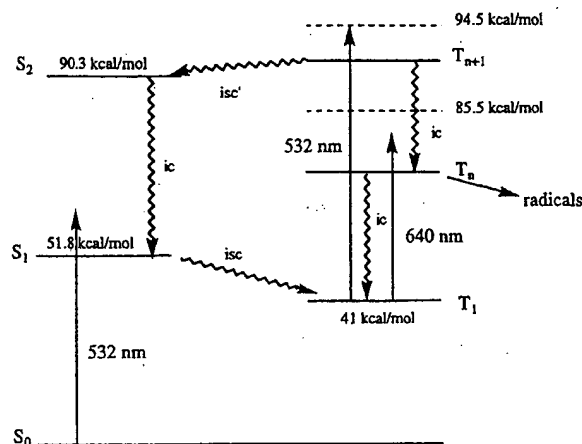


Figure 7. Proposed energy level diagram for RB.

place at shorter wavelengths ($\lambda < 575$ nm) including 532 nm. Detection of fluorescence emission concomitant with the second pulse provides evidence for the occurrence of pathway iii. This fluorescence emission is spectrally identical to normal fluorescence emission of RB, indicating that the S_1 state is accessed by reverse intersystem crossing from upper T_n states to the singlet manifold and subsequent internal conversion. In the 532 + 640-nm case, the emission is only seen when both laser pulses are used; irradiation of the sample with a single 640-nm pulse produces no emission, as expected.

The observation that the photobleaching of the T_1 state was reduced as the wavelength of the second pulse approached the ground state absorption of RB (Figure 5) was of initial concern. Thus, steps were taken to ensure that the apparent lack of photobleaching was not simply due to a secondary ground state excitation and triplet formation that had the effect of compensating for the bleaching of the T_1 state. A number of different excitation geometries were used to ensure maximum overlap of the laser beams and homogeneity of the two lasers. In addition, complete conversion of RB to the T_1 state in the first 532-nm pulse was routinely employed. The absorption coefficient of the S_0 state is only a factor of 2 greater than that of T_1 at this wavelength,¹⁰ and under complete conversion conditions it can be seen that $<10\%$ of the triplet state has decayed prior to the second pulse, indicating that the absorption of the second pulse is almost exclusively by the T_1 state. Under these conditions, the magnitude of the transient absorption was exactly the same following both pulses, showing that no bleaching of the T_1 state took place on 532 + 532-nm excitation. This is to be contrasted with the analogous 532 + 640-nm experiment where $\sim 30\%$ bleaching was typical.

Thus, it appears that the photochemistry of the T_1 state is indeed wavelength-dependent. Possible excited state processes for sequential two-photon excitation of RB are summarized in Figure 7. For wavelength-dependent photochemistry to occur, at least two electronic excited states with different properties must be accessed. Excitation of T_1 with 640 nm is depicted as accessing an upper state T_n whereas 532-nm excitation of T_1 gives a higher state T_{n+1} . The lack of photobleaching of T_{n+1} requires that a nonradiative relaxation pathway should exist from this level but not from T_n . Internal conversion is therefore ruled out and the observed reverse intersystem crossing (as seen from fluorescence emission) is a probable candidate. Indeed, a theoretical and experimental study of 532-nm pulsed excitation of RB in solution led to the estimate of a quantum yield of 0.8 for this reverse intersystem crossing process.¹⁸ Our observed

lack of photobleaching in the 532 + 532-nm experiment is consistent with this interpretation; S_1 - T_1 intersystem crossing is so efficient that even if as much as 80% of the T_1 state is converted to S_1 via reverse intersystem crossing from T_{n+1} , quantitative repopulation of the T_1 state in a time scale shorter than the laser pulse occurs and no bleaching is observed following the pulse. This mechanism also accounts for the much greater fluorescence emission observed using 532 nm compared to 640 nm as the second pulse. A similar, highly efficient reverse intersystem crossing from upper triplet states was reported by us for merocyanine dye derivatives ($\Phi_{isc} = \sim 0.75^{13}$), where the mechanism was more readily apparent on two-photon, two-color laser flash photolysis, due to the lower quantum yields of S_1 - T_1 intersystem crossing in these compounds that produced observable bleaching of the T_1 state by the second pulse.

Figure 7 shows an energy level scheme that accounts for the observed photochemistry. Absorption of a 640-nm photon (44.5 kcal/mol) by the lowest triplet state ($E_T \sim 41$ kcal/mol^{15,19-21}) raises the molecule to a level of 85.5 kcal/mol above the ground state (S_0). Similarly, absorption of a 532-nm photon (53.5 kcal/mol) by T_1 accesses a level of 94.5 kcal/mol above S_0 . Rose Bengal has a UV absorption band at 315 nm, equivalent to an energy of 90.4 kcal/mol. Thus, the T_n state populated by the 640-nm photon must be lower in energy than this (presumably) S_2 state, whereas enough energy is coupled in via a 532-nm photon to reach a T_{n+1} state at an energy similar to the S_2 state and from which reverse intersystem crossing may occur. Such a route is not open to the T_n state that can either relax by internal conversion or undergo bleaching and radical formation. The fact that bleaching is not observed on 532-nm excitation suggests that the rate constant for reverse intersystem crossing (k_{isc}) dominates the relaxation from the T_{n+1} state, in agreement with the reported quantum yield of reverse intersystem crossing of 0.8 on absorption of a 532-nm photon by the T_1 state.¹⁸ Presumably, a small energy gap between T_{n+1} and S_2 is responsible for the dominance of this pathway over internal conversion in the triplet manifold and to repopulate T_1 .

These results indicate that differences in RB-photosensitized inhibition of ACE by sequential two-photon absorption in a single, high-intensity 532-nm pulse compared to two-color excitation with 532 + 640-nm pulses result from different upper triplet states reached by 532 or 640-nm excitation of the T_1 state in these experimental systems. The radicals produced via 640-nm excitation of T_1 to T_n give rise to oxygen-independent photosensitization,⁵ but in the high-intensity, single pulse experiment, the T_{n+1} state decays by reverse intersystem crossing to S_2 and ultimately returns to T_1 via intersystem crossing from S_1 , with the result that no additional reactive intermediates are formed and only the T_1 -mediated formation of singlet oxygen is responsible for photosensitized inhibition of ACE.¹⁰

These results reaffirm the fact that two-photon chemistry is just as selective as one-photon chemistry and is not just a matter of the amount of excess energy that is absorbed by the T_1 state. The result here is anti-intuitive, as we expect bond cleavage to arise from a dissociative state and that excess energy would enhance the effect. However, in the case of RB the photochemistry of the triplet state is dependent on the relative positions of upper singlet and triplet states.

Acknowledgment. This work was supported by NIH RO1 CA68524 (R.W.R.), NIH GM30755 (I.E.K.) and ONR contract N00014-96-1-0927. We would like to thank Hans-Christian Luedemann for technical assistance and Tom Foster and John Larkin, University of Rochester, for helpful discussions.

References and Notes

- (1) Andreoni, A.; Cubeddu, R.; De Silvestri, P.; Laporta, P.; Svelto, O. *Chem. Phys. Lett.* **1982**, *88*, 37–39.
- (2) Andreoni, A. *J. Photochem. Photobiol. B: Biol.* **1987**, *1*, 187–193.
- (3) Motten, A. G.; Buettner, G. R.; Chignell, C. F. *Photochem. Photobiol.* **1985**, *42*, 9–15.
- (4) Buettner, G. R.; Hall, R. D.; Chignell, C. F.; Motten, A. G. *Photochem. Photobiol.* **1989**, *49*, 249–256.
- (5) Smith, G.; McGimpsey, W. G.; Lynch, M. C.; Kochevar, I. E.; Redmond, R. W. *Photochem. Photobiol.* **1994**, *59*, 135–139.
- (6) Stiel, H.; Teuchner, K.; Paul, A.; Freyer, W.; Leupold, D. *J. Photochem. Photobiol. A: Chem.* **1994**, *80*, 289–298.
- (7) Garcia, C.; Smith, G. A.; McGimpsey, W. G.; Kochevar, I. E.; Redmond, R. W. *J. Am. Chem. Soc.* **1995**, *117*, 10871–10878.
- (8) Stiel, H.; Teuchner, K.; Leupold, D.; Kochevar, I. E. *J. Photochem. Photobiol. B: Biol.* **1996**, *33*, 245–254.
- (9) Allen, M. T.; Lynch, M.; Lagos, A.; Redmond, R. W.; Kochevar, I. E. *Biochim. Biophys. Acta* **1991**, *1075*, 42–49.
- (10) Lambert, C. R.; Stiel, H.; Leupold, D.; Lynch, M.; Kochevar, I. E. *Photochem. Photobiol.* **1996**, *63*, 154–160.
- (11) Lachish, U.; Shafferman, A.; Stein, G. *J. Chem. Phys.* **1976**, *64*, 4205–4211.
- (12) Scaiano, J. C.; Johnston, L. J.; McGimpsey, W. G.; Weir, D. *Acc. Chem. Res.* **1988**, *21*, 22–29.
- (13) Redmond, R. W.; Kochevar, I. E.; Krieg, M.; Smith, G.; McGimpsey, W. G. *J. Phys. Chem.* **1997**, *101*, 2773–2777.
- (14) Aveline, B.; Hasan, T.; Redmond, R. W. *Photochem. Photobiol.* **1994**, *59*, 328–335.
- (15) Murasecio-Suardi, P.; Gassman, E.; Braun, A. M.; Oliveros, E. *Helv. Chim. Acta* **1987**, *70*, 1760–1773.
- (16) Fleming, G. R.; Knight, A. W. E.; Morris, J. M.; Morrison, R. J. S.; Robinson, G. W. *J. Am. Chem. Soc.* **1977**, *99*, 4306–4311.
- (17) Bensasson, R. V.; Goldschmidt, C. R.; Land, E. J.; Truscott, T. G. *Photochem. Photobiol.* **1978**, *28*, 277–281.
- (18) Reindl, S.; Penzkofer, A. *Chem. Phys.* **1996**, *211*, 431–439.
- (19) Shen, T.; Zhao, Z.-G.; Yu, Q.; Xu, H.-J. *J. Photochem. Photobiol. A: Chem.* **1989**, *47*, 203–212.
- (20) Wintgens, V.; Scaiano, J. C.; Linden, S. M.; Neckers, D. C. *J. Org. Chem.* **1989**, *54*, 5242–5246.
- (21) Redmond, R. W. *Photochem. Photobiol.* **1991**, *54*, 547–556.

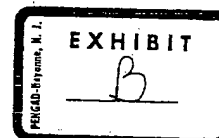


SENT BY:ARNOLD, WHITE&DURKEE

8- 2-95 : 2:03PM ;

7137892679+

2



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

JOHN KHADEM
TOAN VINH TRUONG

Serial No.: 08/033,314

Filed: March 15, 1993

For: ORGANIC TISSUE GLUE FOR
CLOSURE OF WOUNDS

§
§
§
§
§
§
§
§
§
§
§

Group Art Unit: 1507

Examiner: S. Berman

Attorney Docket: ARCD077/KAM

DECLARATION OF JOHN KHADEM, M.D.

1. I, John Khadem, M.D. do hereby declare and state that I am a citizen of the United States of America and that I reside at 3 Sagamore #3, Quincy, Massachusetts, 02171. I received a Bachelor of Arts in Chemistry and Sociology from Duke University in 1987 and a Master of Public Health degree from Columbia University, School of Public Health in 1991. I received by M.D. from Columbia University, College of Physicians and Surgeons.
2. I am a co-inventor of United States patent application identified above.
3. I am familiar with the articles cited by the Examiner in an Office Action dated April 4, 1995: (1) Goosey et al., "Crosslinking of Lens Crystallins in Photodynamic System: A Process Mediated by Singlet Oxygen"; (2) Goosey et al., "Effects of Singlet Oxygen on Human Lens Crystallins in Vitro"; (3) Andley, "Spectroscopic Studies on the Riboflavin-Sensitized Conformational Changes of Calf Lens Alpha-Crystallin"; (4) Girotti et al., "Methylene Blue-Sensitized Photooxidation of Hemoglobin: Evidence for Cross-Link

BEST AVAILABLE COPY

Formation"; (5) Wider et al., "Skin Closure with Dye-Enhanced Laser Welding and Fibrinogen"; and (6) Bass et al., WO, A, 92/02238.

4. I have performed experiments to demonstrate that compositions below 1% protein (1% = 10 mg/ml) are not sufficiently viscous to produce adhesive properties upon photoactivation.

5. The experiments were conducted using the following materials obtained from Sigma Chemical: fibrinogen, riboflavin-5-phosphate (RSP), alpha crystalline and beta (h) crystalline.

6. The experiments were designed as follows:

Experiment 1. The first formulation contained 2.6 mg of RSP dissolved in 1.0 cc of 18% fibrinogen in de-ionized water. Ten eyes were used in this experiment.

Experiment 2. The second formulation contained 18 mg of fibrinogen, 2.6 mg of RSP and 2.0 cc of de-ionized water. This yielded a protein concentration of 0.9% fibrinogen. One eye was used in this experiment.

Experiment 3. The third formulation contained 0.8 mg of alpha crystalline, 1.0 mg of RSP, and 0.3 cc of de-ionized water. This yielded a protein concentration of 0.27% alpha crystalline similar to the 2.4 mg/ml as per Goosey et al. One eye was used in this experiment.

Experiment 4. The fourth experiment contained 0.8 mg of beta (h) crystalline, 1.0 mg of RSP, and 0.3 cc of de-ionized water. This yielded a protein

concentration of 0.27% beta (h) crystalline. One eye was used in this experiment.

7. All formulation mixtures of Experiments 1-4 were made within two hours of use. The formulation container was then wrapped in aluminum foil until ready for use. Frozen, non-preserved, human cadaver eyes were defrosted in room temperature water. An 18 gauge butterfly needle was connected with a water bottle. The bottle's inner pressure was controlled by a hand-pumped sphygmomanometer. The needle was inserted in the vitreous cavity through equatorial sclera and the eye was pressurized to 25-30 mm Hg. The corneal epithelium was then removed at the wound site. A caliper set on 5 mm was used to mark the extent of the incision across central cornea. A simple through-and-through incision was made with a disposable sharp blade. The anterior chamber was then reformed with air. The formulation mixtures of Experiments 1-4 were applied in a layer to the surface of the wound with a TB syringe and a 30 gauge needle. A small amount was then injected within the wound throughout its extent.

8. Argon laser (488-514 nm, HGM Argon) at a setting of 0.6 watts, 2 mm spot size was applied to the wound for 2 minutes in a continuous back and forth manner using the hand-held fiber optic probe. The sphygmomanometer was then pressurized in approximately 10 mm Hg increments. Bursting pressure was recorded the moment the wound leaked air or intraocular fluid.

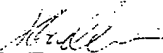
9. The bursting pressures obtained for the formulations of Experiments 1-4 along with the standard deviations are presented below:

Experiment Number	Mean Bursting Pressure	Standard Deviation
1	154 mm Hg	55 mm Hg
2	0.0 mm Hg	-
3	0.0 mm Hg	-
4	0.0 mm Hg	-

10. It is evident that the formulation solutions of Experiments 2-4 comprising less than 1% protein completely failed to provide any adhesive properties (i.e. leak pressure was 0.0 mm Hg). On the other hand, the formulation solution of Experiment 1 comprising over 1% protein produced substantial adhesive properties.

I declare all statements made herein of my own knowledge are true and that all statements made on information and belief or believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

8/2/95
Date


John Khadem, M.D.

FAUCD077PTOKHADEM.DEC

Serial No. 08/033,314
Declaration of John Khadem, M.D.

BEST AVAILABLE COPY